AD________________

Award Number: DAMD17-01-1-0700

TITLE: Evaluation of the role of the metastasis-suppressor gene MKK4/SEK1 in transgenic models of prostate cancer

PRINCIPAL INVESTIGATOR: Carrie W. Rinker-Schaeffer, Ph.D.

CONTRACTING ORGANIZATION: The University of Chicago
Chicago, IL 60637

REPORT DATE: June 2005

TYPE OF REPORT: Final, Revised

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*
01/06/05

2. REPORT TYPE
Final, Revised

3. DATES COVERED *(From - To)*
1 Jun 2001 – 31 May 2005

4. TITLE AND SUBTITLE

Evaluation of the role of the metastasis-suppressor gene MKK4/SEK1 in transgenic models of prostate cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-01-1-0700

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Carrie W. Rinker-Schaeffer, Ph.D.

E-Mail: crinkers@midway.uchicago.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Chicago
Chicago, IL 60637

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: Metastasis-suppressor genes suppress the growth of metastases without affecting tumor growth. We have been studying the role of inactivation of one such metastasis suppressor gene, Map Kinase Kinase 4 (MKK4) in the process of metastatic colonization. Work proposed in this application was aimed at extending our ongoing studies in the AT6.1 model system into established transgenic models of prostate cancer and the use of transgenic approaches to test further MKK4's metastasis suppressor activity. Over the finding period of this grant we worked to test our hypotheses appropriately. During the course of these studies we found a need for changing our experimental design. To address these we established new collaborations and re-examined the potential role of MKK4 in tumorigenesis in both the TRAMP model and human cancers. In pursuing these studies we identified an unanticipated role MKK4 in the early growth of primary tumors and disseminated cells at metastatic sites.

15. SUBJECT TERMS
No subject terms provided

16. SECURITY CLASSIFICATION OF: a. REPORT	b. ABSTRACT	c. THIS PAGE	17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
U	U	U	UU	38	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

1. Introduction

This year 560,000 Americans will die from cancer. The majority of these patients will die from the consequences of metastatic disease (1). Surgery and adjuvant therapies effectively control many localized cancers yet options for treating metastatic disease are limited and, for certain cancers such as prostate, only marginal improvements in patient survival have been made. Although many prostate cancers are believed to be organ-confined at the time of definitive local therapy, the majority of patients likely harbor viable tumor cells that have escaped from the primary tumor and reside at secondary sites. The prostate cancer recurrence rate after radical prostatectomy or radiotherapy is approximately 20-40% (2). Prostate cancer is just one example of the wider need for rational biological and molecular targets that effectively identify patients at risk for relapse, and for discovering avenues for therapeutic intervention.

Cancer metastasis is a complex, dynamic process which begins with the dissemination of cells from the primary tumor and culminates in the formation of clinically detectable, overt metastases at one or more discontinuous secondary sites. Presence of a primary tumor is a requirement for metastasis. The impairment of any individual step in this cascade of *in vivo* events may thwart the overall process. *Metastatic colonization* is the final step in this process and is defined as the lodging and subsequent growth of disseminated cancer cells into detectable metastases (Fig. 1) (3, 4). Multiple lines of evidence have shown that cancer cells can be shed very early within the time course of primary tumor growth. Studies using polymerase chain reaction (PCR) and immunohistochemical approaches have detected cancer cells in the circulation and at secondary sites despite the fact that the primary tumor was localized and metastases could not be detected clinically. Additionally, the lodging and survival of single cells at secondary sites is a more efficient process than once believed (3). Collectively, these data suggest that cancer cell dissemination is an early event and that cells may persist in a quiescent state.

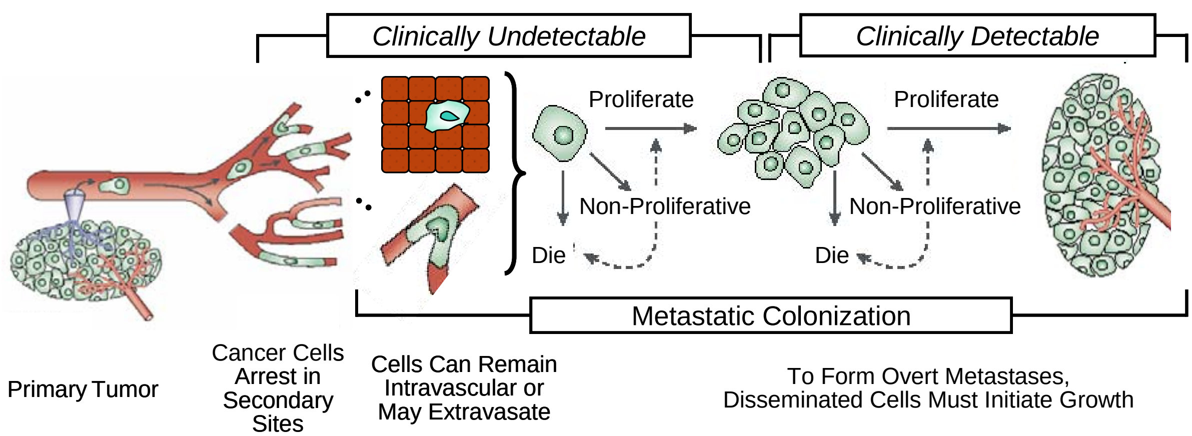

Fig. 1. Metastatic colonization is the final step in the development of metastases. After lodging at secondary sites, cells can either remain intravascular or extravasate into surrounding tissues. To form detectable metastases, disseminated cancer cells must activate signaling cascades, enabling them to survive, enter the cell cycle, and divide. Progressive growth requires the fraction of proliferating cells to exceed the fraction of cells that are non-dividing or apoptotic. The process is likely regulated by specific signaling cascades that are activated by complex interactions between cancer cells and their microenvironment. [Adapted from (3)]

Clinically and experimentally, primary tumor formation and metastasis are distinct processes which share some common features such as control of proliferation. Locally growing tumors can progress without the development of overt metastases, suggesting that the molecular processes regulating tumorigenicity and metastasis are distinct and can be targeted therapeutically. During the process of transformation and subsequent progression to a malignant phenotype, genetic and epigenetic factors alter the ability of a cell to perceive and respond to signals that regulate normal tissue homeostasis. A minority of tumor cells accrue the full complement of alterations that enables them to disseminate from the primary tumor, survive attacks from the immune system and biophysical forces, respond to growth-promoting and/or inhibitory signals from the distant tissues, and thrive. Once disseminated cells gain the ability to initiate proliferation it is possible that they will exhibit phenotypic characteristics similar to early stages of primary tumor formation. Studies of proteins that specifically inhibit the ability of cells to form metastases are providing new insights into the molecular mechanisms regulating these complex processes (5).

Identification of metastatic colonization as an important therapeutic target was largely due to the effort of several laboratories to identify metastasis suppressors (5, 6). *Metastasis suppressors* are operationally defined as specifically regulating metastasis formation *without* affecting primary tumor growth animal models of metastasis (5). Many metastasis suppressors would not have been predicted *a priori* based upon their previously known cellular function(s) (4). To date, our laboratory and others have identified 11 gene products with *bona fide* metastasis suppressor activity that regulate one or more steps of metastasis, including metastatic colonization (5). Specifically, *in vivo* studies demonstrate that metastatic cancer cells which express ectopic MKK4, KISS1, nm23-H1, or SSeCKs proteins can disseminate and lodge at secondary sites, but are suppressed in their ability to colonize target tissues (i.e. form overt metastases) in experimental exnograft models (7-12).

Since 1990, the PI has participated in an organized effort to identify metastasis suppressors. In 1999, her laboratory identified mitogen-activated protein kinase kinase 4/c-Jun NH2-terminal kinase-activating kinase/stress-activated protein/Erk kinase 1 (hereafter referred to as *MKK4*), as prostate cancer metastasis suppressor. The identification of metastasis suppressor activity requires the use of reproducible, quantitative *in vivo* models of metastasis. Cancer cell lines must be sufficiently metastatic to ascertain suppression of metastasis formation and be susceptible to genetic manipulation and biochemical studies. Furthermore, the models must have an established literature correlating findings from animal studies with clinical disease and be amenable to screening gene/protein functions rapidly. We initially used the AT6.1 Dunning rat prostatic cancer model for our functional studies because they best meet the requirements listed above. Clearly, however this model has limitation inherent to all xenograft models, thus we proposed to test the function of MKK4 in additional transgenic models of prostate cancer.

MAP kinases occupy a central position in cell growth, differentiation, and transformation (Fig. 2). To date, three MAP kinase modules have been well-characterized: extracellular signal-regulated protein kinase (ERK), c-Jun NH2-terminal protein kinase (JNK), and p38 (13). Each cascade consists of a MAP3K, a MAP2K, and a MAPK. The ERK pathway is activated predominantly by mitogenic stimuli via Raf and MEK1, the MAP3K and MAP2K, respectively. The substrates of activated ERK include transcription factors such as c-Fos and Elk-1. In contrast, the JNK and p38 pathways are generally activated in response to stress stimuli. MKK4 is a dual-specificity serine/threonine kinase within the SAPK cascade, which consists of the c-Jun NH2-terminal protein kinase (JNK) and p38 signaling. The JNK signaling cascade consists of two MAP2Ks, MKK4 and MKK7, while the p38 signaling cascade MAP2Ks include JNKK1, MKK3, and MKK6. MEKK1 is one of several MAP3Ks that are able to strongly activate both JNKK1 and MKK7 (14, 15). The MAP3Ks that specifically activate p38 are not well defined but may include TAK1 (16, 17). The substrates of activated JNK and p38 include transcription factors such as ATF-2 and c-Jun (13, 18, 19). Therefore, activation of either of these pathways can initiate dramatic changes in gene expression. The biological outcome of MAPK activation depends on a variety of factors including cell type, cell environment, signal strength and duration, and subcellular localization of signaling proteins.

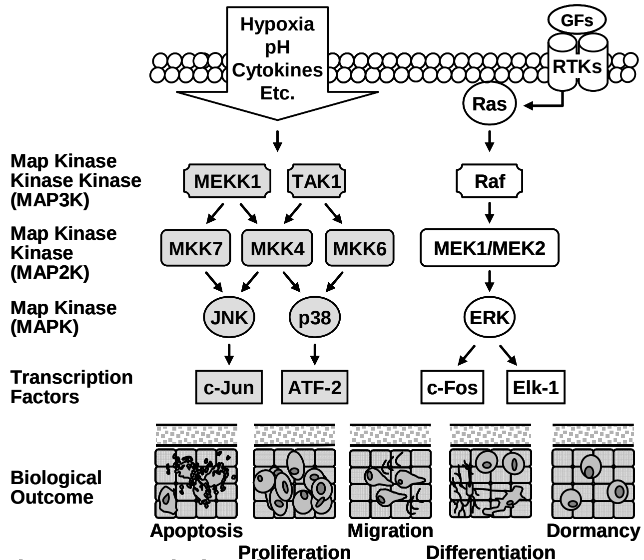

Fig. 2. MKK4 is involved in SAPK signaling. MKK4 can activate the JNK ag modules (25). The JNK and p38 pathways are generally activated in response to stress stimuli p38 MAP kinases. Components of the SAPK pathway are shaded in gray.

Using the AT6.1 model in combination with positional cloning we identified a metastasis suppressor function for MKK4 (7). The biological effect of ectopic expression of MKK4 and components of its signaling cascade on the formation of spontaneous AT6.1 metastases is illustrated by representative *in vivo* data in Fig. 3. Expression of MKK4 reduced the number of overt surface lung metastases. Studies using the kinase inactive MKK4 mutant (MKK4-KR) further demonstrated that the kinase activity of MKK4 is required for suppression of overt metastases and is sufficient to prolong animal survival (8). Ectopic expression of MKK7, a JNK-specific kinase, suppresses the formation of metastases, whereas MKK6, a p38-specific kinase, has no effect.

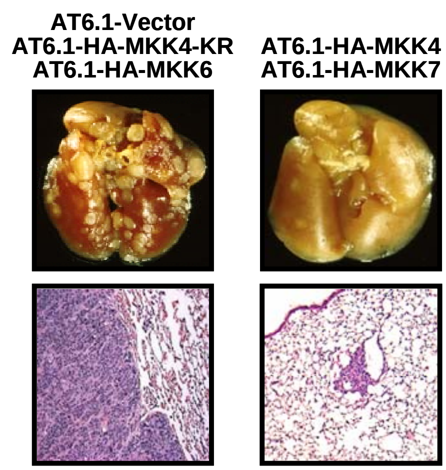

Fig. 3. Summary of the metastasis suppressor activities of MKK4, MKK4-KR, MKK6 and MKK7 in the AT6.1 model system. Lungs harvested from tumor-bearing animals injected with AT6.1 cells expressing indicated constructs (42 days post injection). ***Upper*** surface metastases, ***Lower*** histological metastases.

Metastasis-suppressed AT6.1-HA-MKK4 cells are able to form microscopic foci within the mouse lung, suggesting that MKK4-mediated metastasis suppression is not blocking tumor cell invasion and dissemination but rather suppressing the ability of cells to undergo expansion at secondary sites (8). In support of this model, quantitative studies found comparable numbers of JNKK1-expressing and control cells lodged at secondary sites at early time points during metastatic colonization (8). The known targets of activated MKK4, the p38 and JNK MAPKs, have an established role in stress-activated signaling can result in induction of apoptosis, raising the possibility that decreased expansion is due to an increased percentage of JNKK1-expressing cells undergoing cell death. Evaluation of disseminated JNKK1-expressing cells and microscopic metastases in the AT6.1 model showed very low rates of apoptosis in both metastatic and metastasis suppressed cells (unpublished data). These data imply activated JNKK1 impairs the ability of disseminated cells to initiate proliferation and/or proceed through the cell cycle.

A rich and growing literature support a role for both p38 and JNK in cell survival, cell cycle regulation, and proliferation. JNK has an established function in the G1/S transition as well as an emerging role in G2/M progression and cytokinesis (20). The involvement of p38 in cell cycle progression is more complex, activating cell cycle checkpoints in some systems and promoting progression through the cell cycle in others (21). For example, p38 has been implicated in promoting the G2/M checkpoint, delaying entry into mitosis via Cdc25b inhibition. Furthermore, p38 activation can or may prevent anaphase entry by activating the spindle assembly checkpoint. These data suggest a role for activated MKK4 in regulation of cell growth. While we considered this effect only at the metastatic site, data presented herein suggests that MKK4 may also be activated in primary tumors in human and TRAMP prostate cancers, thereby regulating growth.

2. Body

As described in the Introduction, we have previously identified a role for the stress-activated protein kinase signal transduction pathway in the suppression of metastatic colonization. Specifically, our laboratory identified MKK4 as a metastasis-suppressor gene encoded by human chromosome 17p11.2. Ectopic expression of MKK4 in highly metastatic Dunning AT6.1 rat prostatic cancer cells suppressed the metastatic ability of the cells by approximately 77%. Using biologic approaches we have recently shown that equal numbers of cells escape from the primary tumor and reach secondary sites further supporting our initial observation that MKK4 metastasis-suppressed cells complete all early steps in the metastatic cascade, but are growth-inhibited at the secondary site. More recently our laboratory has shown that suppression of colonization is dependent on MKK4's kinase activity. In addition, MKK4 appears to signal through the JNK MAPK. While we initially thought that MKK4's activity induced apoptosis, our recent studies suggest that at solitary disseminated cells expressing MKK4 are growth suppressed, but viable. Kim *et al.* found that MKK4 expression was down regulated in clinical prostatic tumors with increased metastatic potential. In collaboration with Yamada and her colleagues we found similar results in ovarian cancer. Specifically down regulated in clinical ovarian cancer metastases and MKK4 expression suppresses metastatic colonization in xenograft models of ovarian cancer. These findings support a role for MKK4 as a metastasis suppressor gene in clinical cancers (8).

The purpose of the work proposed in this DOD Idea Award was to extend our findings from clinical materials and xenograft models into additional prostate cancer models. It has been exceedingly difficult to test metastasis suppressor functions in transgenic systems. For our laboratory and others, using transgenic approaches to dissect the function(s) of metastasis suppressor proteins has been rife with technical problems as well as an incomplete understanding of how the metastasis suppressors were functioning. Indeed, our colleagues studying Brms1 and NM23 have not successfully conducted studies analogous to the ones that we originally proposed. As we conducted the work proposed in our original application we also realized that our operational definition of metastasis suppressor function was incomplete and needed to be reassessed. That is metastasis suppressors were operationally defined as specifically suppressing the formation of overt metastases without appreciable effect on the primary tumor. While this definition was appropriate for the functional studies in xenograft models it implied that somehow these proteins would have a single function and almost switch-like behavior, being on in localized primary tumors and off when cells escaped from the primary site.

As we learned more about the *in vivo* biology of metastasis, we realized that this model did not account for the early dissemination of cancer cells in both animal models and clinical disease. Further, it did not account for the potential for dynamic regulation of a metastasis suppressor such as MKK4, which as a signaling kinase can respond very rapidly to changing environmental conditions throughout steps of the metastatic cascade. To address this complexity we realized that we had to go back to earlier stage TRAMP specimens to test the potential for the dynamic regulation of MKK4 and members of its signaling cascade during the process of tumor progression and metastasis. This new approach nullified the utility of studying the LADY mice which we proposed in our original application. In order to increase the translational relevance of these findings we knew that the TRAMP studies had to be coupled with clinical correlative studies using human tissues. We and our collaborators had additional independent funding and IRB approved protocols to support such a study since they were not part of the work originally proposed in our DOD IDEA Award. DOD funding was not used for the clinical correlative studies. However, coupling of these two synergistic lines of research was essential to answering important biological questions and to make the work of significant impact for publication. Capitalizing on our findings regarding the metastasis suppressor activities of MKK4, MKK6 and MKK7 in the Dunning model we examined the potential role of this signaling module in early events in prostate tumor initiation and progression. Further, we had to restructure our team to address difficulties that we and others were facing with regard to developing transgenic models to test metastasis suppressor gene function. We are delighted to state that work reported in this document is now in press in The Journal of Pathology.

Upregulation of MKK4, MK6 and MKK7 During Prostate Cancer Progression: An Important Role for SAPK Signaling in Prostatic Neoplasia The Stress Activated Protein Kinase (SAPK) signaling pathway culminates in the phosphorylation and activation of the JNK and p38 Mitogen Activated Protein Kinases (MAPKs). These MAPKs activate a variety of essential transcription factors including c-JUN and ATF-2, resulting in diverse, cellular context-dependent outcomes, including apoptosis, proliferation, and differentiation. The role of the MAPK proteins in tumor progression is complex. Historically, JNK activity was thought to be essential for oncogenic

transformation by ras (an oncogene mutated in 30% of human tumors), likely by c-JUN mediated suppression of p53 (22, 23). However, recent *in vitro* studies have suggested that JNK and p38 activity may actually inhibit tumorigenesis in ras-oncogene transformed cells via apoptotic responses and cellular senescence, respectively (24, 25). In fact, JNK-null fibroblasts are not only effectively transformed by ras, but show increased metastatic potential *in vivo* (26). Consistent with this, several recent studies have highlighted an important role for the upstream activators of JNK and p38, the MAPK kinases (MKKs), as inhibitors of tumor progression.

Of all of the MAPK kinases, MKK4 has been best studied in the human carcinoma sequence. MKK4 was initially identified as a tumor suppressor gene because it is mutated and functionally inactivated in ~5% of human cancer cell lines and human tumor tissues, including pancreatic, breast and colon cancer (27-30) Recent studies have extended these results to include ovarian carcinoma, where loss of heterozygosity for MKK4 is present in a majority of cases of the serous papillary subtype (31) Our laboratory originally identified MKK4 as a *metastasis suppressor protein* in two different animal models of metastasis (32, 8, 9). Consistent with this, loss of MKK4 protein expression in human primary tumors, as studied by immunolabeling, has been correlated with shortened patient survival in both gastric and pancreatic carcinoma (33, 34). Additionally, studies of metastatic specimens, including breast, ovarian and pancreatic cancer, have shown decreased MKK4 expression in disseminated cancer cells, either by semi-quantitative RT-PCR or by immunohistochemistry.

Recently, our laboratory extended our initial results to identify all three MAPK kinases, MKK4, MKK6 and MKK7, as metastasis suppressor proteins in animal models of prostate and ovarian cancer metastasis (8, 9) In the well-characterized Dunning AT6.1 model of spontaneous prostate cancer metastasis, ectopic expression of MKK4 or MKK7 reduces lung metastases by 90%. While MKK4 activates both JNK and p38, MKK7 is specific for JNK. Significantly, MKK6, a specific activator of p38, does not inhibit metastasis formation in this system although it does in the ovarian system, underscoring the importance of cellular context in determining the biological outcome of MAPK signaling. Consistent with its role as a metastasis suppressor, in early studies of human prostate carcinoma, MKK4 expression was progressively downregulated in higher Gleason grade tumors [19]. However, the potentially dynamic temporal and spatial expression patterns of MKK4, MKK6 and MKK7 have never been systematically studied in human prostate cancer samples nor have they been correlated with patient survival in this disease.

We set out to study the expression of MKK4, MKK6 and MKK7 in a range of benign and malignant prostate tissues, and to link this data to outcome in human prostate carcinoma. Using the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) model, as well as human prostate cancer specimens, we demonstrate that MKK4, MKK6 and MKK7 are all markedly upregulated during intraepithelial prostatic tumorigenesis. Additionally, we show significant upregulation of all three proteins in TRAMP and human invasive prostate tumors. Interestingly, increased MKK4 and MKK7 expression was associated with higher pathologic stage at prostatectomy, although there was no consistent association with time to biochemical recurrence in multivariate analyses. Overall, these findings uncover a fundamental and cell context-specific role for the MAPK signaling cascade during prostatic tumorigenesis.

Results

MKK4, MKK6 and MKK7 are expressed by prostate cancer cell lines and human prostate tumors by immunoblotting. Immunoblots were performed in order to confirm that MKK4, MKK6 and MKK7 proteins are expressed by human prostate carcinoma, as well as to demonstrate the specificity of the antibodies to be used for immunohistochemistry experiments. Immunoblotting of two human prostate cancer cell lines (PC3 and LNCaP) and three flash-frozen, histologically confirmed samples of human prostate carcinoma revealed that MKK4 (MW = 44 kD), MKK6 (MW = 38 kD) and MKK7 (MW = 52 kD) are expressed by prostate cancer cell lines and human prostatic adenocarcinoma, and that the antibodies to be used for the immunohistochemistry experiments were sufficiently specific for these proteins in human tissue samples (Fig. 1). Immunoblotting also revealed some variability in the expression levels of these proteins between human tumor samples.

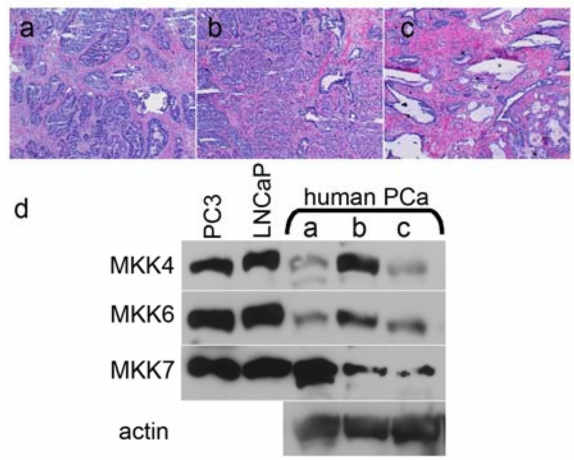

Fig 1. MKK4, MKK6 and MKK7 proteins are expressed in human prostate cancer cell lines and frozen tumor tissue. Frozen sections were prepared from human prostate cancer tissues, and these tissues were subsequently lysed to obtain protein for immunoblotting (all at 40x magnification). **(a)** Gleason grade 7(4+3) **(b)** Gleason grade 7(3+4) and **(c)** Gleason grade 6(3+3) tumors. **(d)** Immunoblotting of 60 µg of protein from each of the above tissues reveals that MKK4 (44 kD), MKK6 (38 kD) and MKK7 (52 kD) are variably expressed in human prostate tumors as well as in 10 µg of lysate from human prostate cancer cell lines (PC3 and LNCaP) and that the antibodies to be used for immunohistochemistry experiments are reasonably specific for these proteins.

MKK4, MKK6, and MKK7 are weakly expressed in mouse and human benign prostatic epithelial cells by immunohistochemistry. Expression of the MAPK kinases was minimal in the prostates of non-transgenic litter-mate control mice, both in the dorsal-ventral prostates as well as in the coagulating gland/anterior prostate (Fig. 2, top panels). Normal human prostatic epithelium adjacent to carcinoma was present for evaluation in 68, 83 and 77 patients for MKK4, MKK6 and MKK7 respectively. All three proteins were only

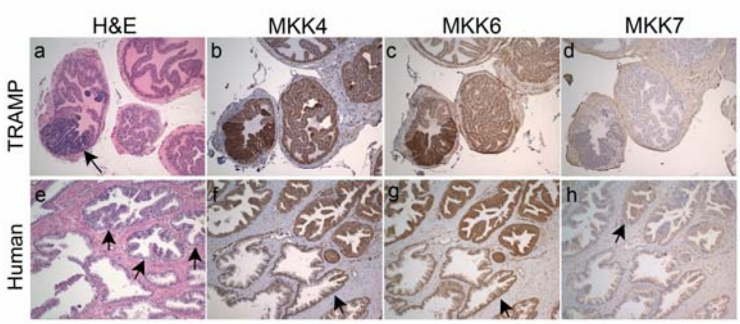

Fig 2: *Increased MKK4, MKK6 and MKK7 expression in TRAMP and human prostatatic intraepithelial neoplasia (PIN).* Representative PIN lesions in both TRAMP and human tissues are characterized by a hyperplastic epithelium, with enlarged nuclei and prominent nucleoli (**a**, **e**, 100x magnification, arrows indicate PIN and high grade PIN in TRAMP and human tissues respectively). MKK4 and MKK6 are overexpressed in TRAMP and human HGPIN relative to surrounding normal prostate epithelial cells (**b**, **c**, **f**, **g** 100x, arrows indicate high MKK4 and MKK6 expression in basal cells of benign human glands). MKK7 is weakly expressed in TRAMP PIN and focally overexpressed in human PIN (arrow) (**d**, **h**, 100x).

weakly expressed in normal prostatic epithelial cells, with immunostaining intensities less than 1+ on average. MKK4 and MKK7 showed predominantly cytoplasmic staining, while MKK6 showed cytoplasmic and nuclear staining in benign prostatic epithelial cells (Fig. 2, lower panels). Significantly, the basal cells showed relatively high intensity staining for all three markers, although this was not a consistent finding in every sample. Stromal and smooth muscle staining was minimal for all MAPK kinases, although skeletal muscle bundles showed staining with MKK7, as expected.

MKK4, MKK6, and MKK7 are upregulated in TRAMP and human HGPIN by immunohistochemistry. MAPK kinase immunostaining was also conducted on TRAMP and human high grade PIN (HGPIN) samples. Most TRAMP mice develop PIN lesions by 12 weeks of age, as evidenced by nuclear enlargement, cellular stratification and crowding. MKK4 and MKK6 expression was dramatically increased in the prostatic glands of mice showing the changes of PIN with nuclear and cytoplasmic localization (Fig 2, top panels). By contrast, in the TRAMP model, MKK7 appeared only modestly upregulated in PIN compared to surrounding benign tissues. In human tissues, all three proteins were significantly upregulated in HGPIN compared to benign glands, with nuclear and cytoplasmic localization (Fig 2, lower panels).

MKK4, MKK6 and MKK7 are significantly upregulated in TRAMP and human prostate cancer relative to benign glands. All three MAPK kinases showed upregulation across a spectrum of TRAMP tumors (Fig 3, upper panels) compared to benign prostatic epithelium. In human tumors, MKK4 and MKK6 were generally highly expressed compared to surrounding benign tissues, with highly significant upregulation of both (Fig 3, lower panels). MKK7 was also upregulated compared to benign tissues, but the intensity difference was significantly smaller. All three markers showed predominantly cytoplasmic localization with a lesser component of nuclear expression. In order to determine the ability of MKK immunostaining to discriminate between benign and malignant glands, ROC analysis was conducted using the mean staining intensity for each patient. MKK4 and MKK6 immunostaining were highly sensitive and specific markers for human prostatic carcinoma (area under the ROC curve = 0.903 and 0.899 respectively), while MKK7 was a less discriminative marker (area under the ROC curve = 0.694).

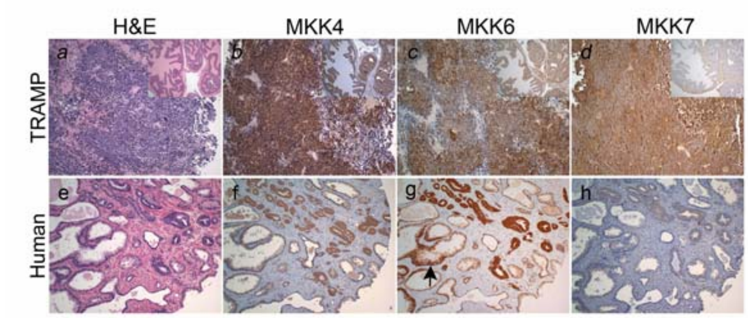

Fig. 3: *Increased MKK4, MKK6 and MKK7 expression in TRAMP and human prostate tumors.* A representative poorly differentiated TRAMP prostate tumor shows increased MKK4, MKK6 and MKK7 expression relative to nearby benign glands from the same animal (inset) (**a**, **b**, **c**, **d** 100x). A representative human prostate tumor (Gleason 6) from the tissue microarray also shows dramatic upregulation of MKK4 and MKK6 with moderate expression of MKK7 in infiltrating malignant glands relative to surrounding, larger benign glands (**e**, **f**, **g**, **h** 100x). A focus of PIN with high expression of MKK6 (**g**, arrow) is coincidentally present.

Increased expression of MKK4, 6 or 7 is significantly associated with some pathologic and clinical variables, but not correlated with time to biochemical recurrence. Finally, we were interested in whether there was any correlation between

clinico-pathologic variables and MAPK kinase staining in the human tumor specimens. Clinical data and follow-up was available on 102 patients. There was no significant correlation with Gleason grade for any of the markers. Higher expression of either MKK4 or MKK7 was significantly correlated with higher pathologic stage at prostatectomy (p=0.006 and 0.015 respectively). Higher MKK6 expression was moderately correlated with lower preoperative PSA (p = 0.035). In multivariate analyses, higher preoperative PSA (p = 0.001), higher Gleason score (p = 0.02), and higher pathologic stage (p<0.001) all predicted for decreased time to biochemical recurrence, as expected. However, increased expression of MKK4, MKK6 or MKK7 was not independently associated with time to biochemical recurrence (p=0.46, p=0.14, p=0.06 respectively).

Evaluating MKK4's metastasis suppressor activity in transgenic models. Our current data from immunohistochemical staining of human and TRAMP prostate cancers, as well as our functional studies in the AT6.1 prostate cancer model, suggest that MKK4's *in vivo* effects are much more dynamic and complex that we initially believed. While our earlier studies of TRAMP tissue suggested a trend in toward correlation between absence of staining and aggressiveness of the primary tumor it was difficult to tease out the expression of MKK4 in matched primary tumors and metatases for technical reasons. We have been able to stain a limited number of metastases and found a low level staining in many samples. Having a large cohort of matched primary tumors and metastases should significantly strengthen the analysis. When this study was originally designed the prevailing view was that metastasis formation was a common event in TRAMP mice. Since then this notion has been show to be incorrect. Metastases are infrequent and those that arise are often neuroendocrine in nature. This has presented a significant problem for accumulating tissues for meaningful analysis. An additional complication that has become apparent is that the background on which the Tag expressed has a profound effect on the extent and type of metastases observed (). The method we proposed to generate metastatic tissues (in our original application) will not yield adequate or appropriate tissues for the study.

Realizing the critical nature of these issues at the time of the last report we established new collaborations with Dr. Barbara Foster (Roswell Park Cancer Center) and Dr. Kent Hunter (NCI). The interaction with Dr. Foster has been fruitful and was key to acquiring the data presented in the preceding sections. Given the data that we have acquired over the past year we are now working with Dr. Foster and Dr. Hunter to determine the best approach to test the effect of MKK4 on the initiation and progression of prostate cancers using transgenic models. Dr. Foster routinely breeds the TRAMP mice onto different genetic backgrounds an will assist in these studies. Dr. Hunter is a mouse geneticist who specific interest is the affect of host genetic background on metastasis. Indeed, during the funding period of this proposal he made novel discoveries regarding the role of genetic background of the mouse strain on its metastasis permissive or suppressive effects. This is completely revolutionary and was not considered at all in our original proposal since it had not yet been discovered. Indeed, in the process of conducting the work originally proposed we and our collaborators have made key discoveries that have affected the approach that we need to take in these studies. As a result of this project we have established a new collaborative team and made significant findings that changed the way we will test MKK4's role in transgenic models.

3. Key Research Accomplishments

We evaluated the expression of MKK4, MKK6 and MKK7 in a large cohort of clinical prostate cancer specimens and TRAMP mouse tissues. We discovered a novel role for MKK4, MKK6, and MKK7 in early phases of prostate cancer tumorigenesis. This work is being submitted for publication and is included as manuscript in the Appendix.

4. Reportable Outcomes

Manuscript - Upregulation of MKK4, MKK6 and MKK7 During Prostate Cancer Progression: An Important Role for SAPK Signaling in Prostatic Neoplasia T L Lotan, M Lyons, D Huo, J Taxy, C Brendler, B Foster, WM Stadler, C. W. Rinker-Schaeffer. *Manuscript being submitted.*

5. Conclusions

The role of SAPK signaling in prostate cancer progression is much more complex than we originally anticipated. That MKK4, MKK6 and MKK7 are all *upregulated* in preneoplastic and neoplastic prostate tissues, and that increased expression of MKK4 or MKK7 is associated with *higher* stage tumors is surprising in light of previous work which has identified these proteins as inhibitors of tumor progression in other contexts. One important caveat is that previous studies have shown that the kinase activity of MKK4 is essential for its ability to inhibit metastatic progression. Because we measured *total expression* of MKK4, MKK6 and MKK7, it is not possible to conclude that MKK4, MKK6 and MKK7 *activity* are truly increased in prostatic tumors. However, several recent *in vivo* studies of JNK and p38 activation in both human and TRAMP prostate cancer support the hypothesis that SAPK activity is significantly upregulated in neoplastic prostate tissues. Uzgare et al found that TRAMP PIN and well-differentiated prostate tumors showed elevated p38 activity (by immunoblot and immunohistochemistry) compared to normal prostate tissues. Similarly, in human prostate cancer samples, two recent studies found upregulation of phospho-JNK, as well as p38 activity (as measured by downstream transcription factors Elk-1 and ATF-2) and MKK6 expression (35-37).

Though mounting evidence points to activation of the SAPK signaling cascade during prostate cancer progression, the biological outcome of this activation remains unclear and is likely cell context-specific. Innumerable studies have linked *in vitro* activation of p38 and JNK with apoptosis in prostate cancer cell lines (38-41). However in TRAMP PIN lesions, *in vivo* p38 activity was associated with cellular proliferation rather than apoptosis[20]. Similarly, though many studies have concluded that activated JNK results in apoptosis in prostate cancer cell lines, others have suggested that this kinase is a potential target for cell growth (39-41). While p38 and JNK activity may be associated with local prostate tumor growth, it is quite possible that activation of the same signaling module in a metastatic tumor could be detrimental to tumor cell survival in that context. Overall, it is most likely that the cellular outcome of SAPK activity depends on the integration of numerous extrinsic signals and intracellular signaling cascades. Recent in vivo studies have suggested that the *balance* of p38 and ERK activity is critical in determining the proliferative fate of tumor cells (42, 43). The fact that increased levels MKK4, MKK6 and MKK7 expression were not independently associated with prognosis in prostate cancer in our study may reflect the significance of such a balance.

While SAPK signaling may play a key role in prostate cancer progression, the upregulated expression of MKK4, MKK6 and MKK7 in prostate lesions may also represent a cellular response to stress during the transformation process. We found that MAPK kinase expression is both increased in intraepithelial prostatic neoplasia and later associated with local prostate tumor progression. In other contexts, expression of SAPK signaling components is also temporally dynamic during tumor progression, perhaps reflecting changing levels of cellular stress. In the stomach, MKK4 expression is significantly increased in gastric intestinal metaplasia (generally a response to inflammation) as well as in gastric dysplasia (a pre-neoplastic lesion) compared to surrounding normal glands (44). Yet in gastric cancers, increasing MKK4 expression was associated with better patient survival, suggesting that MKK4 was progressively downregulated in more advanced tumors. This pattern of expression suggests that even before neoplastic transformation takes place, MAPK kinase levels may be increased as a response to surrounding inflammation or other cellular stressors. However, with tumor progression, the MAPK kinase levels may continue to rise (as in prostate cancer) or fall, perhaps reflecting a defective response to external stress (as in gastric tumors).

Ultimately, our finding that multiple components of the SAPK signaling cascade are dramatically upregulated during prostatic tumorigenesis, both in a mouse model as well as in human tissues, has significant diagnostic and therapeutic ramifications. At a minimum, proteins in the SAPK signaling cascade provide an attractive target for future biomarkers and immunohistochemical tests that may play an important role in diagnosis of prostate tumors. Given the conflicting results *in vitro*, future studies should be aimed at pinpointing the biological effects of JNK and p38 activation *in vivo*, both in localized as well as metastatic prostate tumors. The availability of small molecule inhibitors of p38 and JNK activity will be particularly helpful in these studies and may be of future therapeutic benefit. In the end, identification of the signaling cascades that are differentially activated during prostatic tumorigenesis is a crucial step in the search for future molecular targets in this disease.

6. References

1 Steeg, P. S. Tumor metastasis: mechanistic insights and clinical challenges. Nat Med, *12:* 895-904, 2006.

2. Ward, J. F. and Moul, J. W. Rising prostate-specific antigen after primary prostate cancer therapy. Nat Clin Pract Urol, *2:* 174-182, 2005.

3. Chambers, A. F., Groom, A. C., and MacDonald, I. C. Dissemination and growth of cancer cells in metastatic sites. Nature Reviews. Cancer, *2:* 563-572, 2002.

4. Kauffman, E. C., Robinson, V. L., Stadler, W. M., Sokoloff, M. H., and Rinker-Schaeffer, C. W. Metastasis suppression: the evolving role of metastasis suppressor genes for regulating cancer cell growth at the secondary site.[comment]. Journal of Urology, *169:* 1122-1133, 2003.

5. Rinker-Schaeffer CW, O'Keefe JP, Welch DR, and D, T. Metastasis Suppressor Proteins: Discovery, Molecular Mechanisms and Clinical Application. Clinical Cancer Research, in press.

6 Steeg, P. S. PERSPECTIVES ON CLASSIC ARTICLES: Metastasis Suppressor Genes. J Natl Cancer Inst, *96:* E4-, 2004.

7. Yoshida, B. A., Dubauskas, Z., Chekmareva, M. A., Christiano, T. R., Stadler, W. M., and Rinker-Schaeffer, C. W. Mitogen-activated protein kinase kinase 4/stress-activated protein/Erk kinase 1 (MKK4/SEK1), a prostate cancer metastasis suppressor gene encoded by human chromosome 17. Cancer Research, *59:* 5483-5487, 1999.

8. Vander Griend, D. J., Kocherginsky, M., Hickson, J. A., Stadler, W. M., Lin, A., and Rinker-Schaeffer, C. W. Suppression of metastatic colonization by the context-dependent activation of the c-Jun NH2-terminal kinase kinases JNKK1/MKK4 and MKK7. Cancer Res, *65:* 10984-10991, 2005.

9. Hickson, J. A., Huo, D., Vander Griend, D. J., Lin, A., Rinker-Schaeffer, C. W., and Yamada, S. D. The p38 kinases MKK4 and MKK6 suppress metastatic colonization in human ovarian carcinoma. Cancer Res, *66:* 2264-2270, 2006.

10. Leone, A., Flatow, U., King, C. R., Sandeen, M. A., Margulies, I. M., Liotta, L. A., and Steeg, P. S. Reduced tumor incidence, metastatic potential, and cytokine responsiveness of nm23-transfected melanoma cells. Cell, *65:* 25-35, 1991.

11. Xia, W., Unger, P., Miller, L., Nelson, J., and Gelman, I. H. The Src-suppressed C kinase substrate, SSeCKS, is a potential metastasis inhibitor in prostate cancer. Cancer Research, *61:* 5644-5651, 2001.

12. Goldberg, S. F., Miele, M. E., Hatta, N., Takata, M., Paquette-Straub, C., Freedman, L. P., and Welch, D. R. Melanoma metastasis suppression by chromosome 6: evidence for a pathway regulated by CRSP3 and TXNIP. Cancer Research, *63:* 432-440, 2003.

13. Kyriakis, J. M. and Avruch, J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiological Reviews, *81:* 807-869, 2001.

14. Xu, S. and Cobb, M. H. MEKK1 binds directly to the c-Jun N-terminal kinases/stress-activated protein kinases. J Biol Chem, *272:* 32056-32060, 1997.

15. Schlesinger, T. K., Fanger, G. R., Yujiri, T., and Johnson, G. L. The TAO of MEKK. Front Biosci, *3:* D1181-1186, 1998.

16. Hanafusa, H., Ninomiya-Tsuji, J., Masuyama, N., Nishita, M., Fujisawa, J., Shibuya, H., Matsumoto, K., and Nishida, E. Involvement of the p38 mitogen-activated protein kinase pathway in transforming growth factor-beta-induced gene expression. J Biol Chem, *274:* 27161-27167, 1999.

17. Wang, C., Deng, L., Hong, M., Akkaraju, G. R., Inoue, J., and Chen, Z. J. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature, *412:* 346-351, 2001.

18. Pearson, G., Robinson, F., Beers Gibson, T., Xu, B. E., Karandikar, M., Berman, K., and Cobb, M. H. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocrine Reviews, *22:* 153-183, 2001.

19. Ip, Y. T. and Davis, R. J. Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. Curr Opin Cell Biol, *10:* 205-219, 1998.

20. Shim, J., Lee, H., Park, J., Kim, H., and Choi, E. J. A non-enzymatic p21 protein inhibitor of stress-activated protein kinases. Nature, *381:* 804-806, 1996.

21. MacCorkle, R. A. and Tan, T. H. Mitogen-activated protein kinases in cell-cycle control. Cell Biochem Biophys, *43:* 451-461, 2005.

22. Kennedy, N. J., Davis, R. J.: Role of JNK in tumor development. Cell Cycle, **2:** 199, 2003

23. Schreiber, M., Kolbus, A., Piu, F. et al.: Control of cell cycle progression by c-Jun is p53 dependent. Genes Dev, **13:** 607, 1999

24. Sun, P., Yoshizuka, N., New, L. et al.: PRAK Is Essential for ras-Induced Senescence and Tumor Suppression. Cell, **128:** 295, 2007

25. Tront, J. S., Hoffman, B., Liebermann, D. A.: Gadd45a Suppresses Ras-Driven Mammary Tumorigenesis by Activation of c-Jun NH2-Terminal Kinase and p38 Stress Signaling Resulting in Apoptosis and Senescence. Cancer Res, **66:** 8448, 2006

26. Kennedy, N. J., Sluss, H. K., Jones, S. N. et al.: Suppression of Ras-stimulated transformation by the JNK signal transduction pathway. Genes Dev, **17:** 629, 2003

27. Cazillis, M., Bringuier, A. F., Delautier, D. et al.: Disruption of MKK4 signaling reveals its tumor-suppressor role in embryonic stem cells. Oncogene, **23:** 4735, 2004

28. Su, G. H., Hilgers, W., Shekher, M. C. et al.: Alterations in pancreatic, biliary, and breast carcinomas support MKK4 as a genetically targeted tumor suppressor gene. Cancer Res, **58:** 2339, 1998

29. Su, G. H., Song, J. J., Repasky, E. A. et al.: Mutation rate of MAP2K4/MKK4 in breast carcinoma. Hum Mutat, **19:** 81, 2002

30. Teng, D. H., Perry, W. L., 3rd, Hogan, J. K. et al.: Human mitogen-activated protein kinase kinase 4 as a candidate tumor suppressor. Cancer Res, **57:** 4177, 1997

31 Nakayama, K., Nakayama, N., Davidson, B. et al.: Homozygous deletion of MKK4 in ovarian serous carcinoma. Cancer Biol Ther, **5:** 630, 2006

32 Chekmareva, M. A., Kadkhodaian, M. M., Hollowell, C. M. et al.: Chromosome 17-mediated dormancy of AT6.1 prostate cancer micrometastases. Cancer Res, **58:** 4963, 1998

33. Xin, W., Yun, K. J., Ricci, F. et al.: MAP2K4/MKK4 expression in pancreatic cancer: genetic validation of immunohistochemistry and relationship to disease course. Clin Cancer Res, **10:** 8516, 2004

34. Cunningham, S. C., Kamangar, F., Kim, M. P. et al.: MKK4 status predicts survival after resection of gastric adenocarcinoma. Arch Surg, **141:** 1095, 2006

35. Ricote, M., Garcia-Tunon, I., Bethencourt, F. et al.: The p38 transduction pathway in prostatic neoplasia. J Pathol, **208:** 401, 2006

36. Royuela, M., Arenas, M. I., Bethencourt, F. R. et al.: Regulation of proliferation/apoptosis equilibrium by mitogen-activated protein kinases in normal, hyperplastic, and carcinomatous human prostate. Hum Pathol, **33:** 299, 2002

37. Magi-Galluzzi, C., Montironi, R., Cangi, M. G. et al.: Mitogen-activated protein kinases and apoptosis in PIN. Virchows Arch, **432:** 407, 1998

38. Cho, S. D., Ahn, N. S., Jung, J. W. et al.: Critical role of the c-JunNH2-terminal kinase and p38 mitogen-activated protein kinase pathways on sodium butyrate-induced apoptosis in DU145 human prostate cancer cells. Eur J Cancer Prev, **15:** 57, 2006

39. Curtin, J. F., Cotter, T. G.: Anisomycin activates JNK and sensitises DU 145 prostate carcinoma cells to Fas mediated apoptosis. Br J Cancer, **87:** 1188, 2002

40. Tanaka, Y., Gavrielides, M. V., Mitsuuchi, Y. et al.: Protein kinase C promotes apoptosis in LNCaP prostate cancer cells through activation of p38 MAPK and inhibition of the Akt survival pathway. J Biol Chem, **278:** 33753, 2003

41. Wu, J., Haugk, K., Plymate, S. R.: Activation of pro-apoptotic p38-MAPK pathway in the prostate cancer cell line M12 expressing a truncated IGF-IR. Horm Metab Res, **35:** 751, 2003

39. Godoy-Tundidor, S., Cavarretta, I. T., Fuchs, D. et al.: Interleukin-6 and oncostatin M stimulation of proliferation of prostate cancer 22Rv1 cells through the signaling pathways of p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase. Prostate, **64:** 209, 2005

40. Koochekpour, S., Sartor, O., Hiraiwa, M. et al.: Saposin C stimulates growth and invasion, activates p42/44 and SAPK/JNK signaling pathways of MAPK and upregulates uPA/uPAR expression in prostate cancer and stromal cells. Asian J Androl, **7:** 147, 2005

41. Uzgare, A. R., Isaacs, J. T.: Enhanced redundancy in Akt and mitogen-activated protein kinase-induced survival of malignant versus normal prostate epithelial cells. Cancer Res, **64:** 6190, 2004

42. Aguirre-Ghiso, J. A., Ossowski, L., Rosenbaum, S. K.: Green fluorescent protein tagging of extracellular signal-regulated kinase and p38 pathways reveals novel dynamics of pathway activation during primary and metastatic growth. Cancer Res, **64:** 7336, 2004

43. Ranganathan, A. C., Adam, A. P., Aguirre-Ghiso, J. A.: Opposing roles of mitogenic and stress signaling pathways in the induction of cancer dormancy. Cell Cycle, **5:** 1799, 2006

44. Cunningham, S. C., Kamangar, F., Kim, M. P. et al.: Claudin-4, mitogen-activated protein kinase kinase 4, and stratifin are markers of gastric adenocarcinoma precursor lesions. Cancer Epidemiol Biomarkers Prev, **15:** 281, 2006

7. Appendices

.

Upregulation of MKK4, MKK6 and MKK7 During Prostate Cancer Progression: An Important Role for SAPK Signaling in Prostatic Neoplasia

T L Lotan[1], M Lyons[2], D Huo[3], J Taxy[1], C Brendler[2], B Foster[4], WM Stadler[2,5], C. W. Rinker-Schaeffer[2].

[1]Department of Pathology, University of Chicago, Chicago, IL; [2] Section of Urology, Department of Surgery, University of Chicago, Chicago, IL; [3]Health Studies, University of Chicago, Chicago, IL; [4]Roswell Park Cancer Institute, Buffalo, NY and [5] Department of Medicine, University of Chicago, Chicago, IL

Key words: Prostate cancer, PIN, TRAMP, MKK4, MKK6, MKK7

Corresponding author:
Carrie W. Rinker-Schaeffer, Ph.D.
Section of Urology, Department of Surgery
The University of Chicago
Section of Urology, Department of Surgery, MC6038
crinkers@midway.uchicago.edu
(773) 702-5882

ABSTRACT

Purpose: The Stress Activated Protein Kinase (SAPK) signaling cascade culminates in the phosphorylation of the JNK and p38 MAPKs. Recently, the upstream activators of these proteins, the MAPK kinases (MKKs), have been implicated as inhibitors of tumor progression in a variety of clinical and experimental tumor models. This study evaluates MKK4, MKK6 and MKK7 expression during prostate cancer (PCa) progression in humans and in the TRAMP transgenic mouse.

Materials and Methods: Benign prostate, prostatic intraepithelial neoplasia, (PIN), and tumor tissues were collected from 37 TRAMP mice. Six tissue microarrays were constructed with tumors from a matched group of 102 men undergoing radical prostatectomy. Tissues from an additional 20 patients with extensive high grade PIN (HGPIN) were also analyzed. For all samples, immunohistochemical staining for MKK4, MKK6 and MKK7 was scored in normal and neoplastic glands.

Results: MKK4, MKK6 and MKK7 were all significantly upregulated in HGPIN and PCa when compared to surrounding normal glands in both the TRAMP and human samples ($p<0.0001$ for all markers). Increased expression of MKK4 or MKK7 correlated with higher pathologic stage at prostatectomy ($p=0.006$ and $p=0.015$). In multivariate analysis, there was no association between protein levels and time to biochemical recurrence.

Conclusion: The dramatic upregulation of MKK4, MKK6 and MKK7 during PCa progression in both the TRAMP and human tissues highlights a fundamental role for SAPK signaling in prostatic tumorigenesis. The finding that higher MKK4 and MKK7 expression was associated with higher stage tumors was unexpected and underscores the importance of cellular context in MAPK signaling.

INTRODUCTION

The Stress Activated Protein Kinase (SAPK) signaling pathway culminates in the phosphorylation and activation of the JNK and p38 Mitogen Activated Protein Kinases (MAPKs). These MAPKs activate a variety of essential transcription factors including c-JUN and ATF-2, resulting in diverse, cellular context-dependent outcomes, including apoptosis, proliferation, and differentiation. The role of the MAPK proteins in tumor progression is complex. Historically, JNK activity was thought to be essential for oncogenic transformation by ras (an oncogene mutated in 30% of human tumors), likely by c-JUN mediated suppression of p53 [1, 2]. However, recent *in vitro* studies have suggested that JNK and p38 activity may actually inhibit tumorigenesis in ras-oncogene transformed cells via apoptotic responses and cellular senescence, respectively[3, 4]. In fact, JNK-null fibroblasts are not only effectively transformed by ras, but show increased metastatic potential *in vivo* [5]. Consistent with this, several recent studies have highlighted an important role for the upstream activators of JNK and p38, the MAPK kinases (MKKs), as inhibitors of tumor progression.

Of all of the MAPK kinases, MKK4 has been best studied in the human carcinoma sequence. MKK4 was initially identified as a tumor suppressor gene because it is mutated and functionally inactivated in ~5% of human cancer cell lines and human tumor tissues, including pancreatic, breast and colon cancer[6-9]. Recent studies have extended these results to include ovarian carcinoma, where loss of heterozygosity for MKK4 is present in a majority of cases of the serous papillary subtype[10]. Our laboratory originally identified MKK4 as a *metastasis suppressor protein* in two different animal models of metastasis[11-13]. Consistent with this, loss of MKK4 protein expression in human primary tumors, as studied by immunolabeling, has been correlated with shortened patient survival in both gastric and pancreatic carcinoma[14, 15]. Additionally, studies of metastatic specimens, including breast, ovarian and pancreatic cancer, have shown decreased MKK4 expression in disseminated cancer cells, either by semi-quantitative RT-PCR or by immunohistochemistry [12, 14, 16].

Recently, our laboratory extended our initial results to identify all three MAPK kinases, MKK4, MKK6 and MKK7, as metastasis suppressor proteins in animal models of prostate and ovarian cancer metastasis [17, 18]. In the well-characterized Dunning AT6.1 model of spontaneous prostate cancer metastasis, ectopic expression of MKK4 or MKK7 reduces lung metastases by 90%. While MKK4 activates both JNK and p38, MKK7 is specific for JNK. Significantly, MKK6, a specific activator of p38, does not inhibit metastasis formation in this system although it does in the ovarian system, underscoring the importance of cellular context in determining the biological outcome of MAPK signaling. Consistent with its role as a metastasis suppressor, in early studies of human prostate carcinoma, MKK4 expression was progressively downregulated in higher Gleason grade tumors [19]. However, the temporal and spatial expression patterns of MKK4, MKK6 and MKK7 have never been systematically studied in human prostate cancer samples nor have they been correlated with patient survival in this disease.

We set out to study the expression of MKK4, MKK6 and MKK7 in a range of benign and malignant prostate tissues, and to link this data to outcome in human prostate carcinoma. Using the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) model, as well as human prostate cancer specimens, we demonstrate that MKK4, MKK6 and MKK7 are all markedly upregulated during intraepithelial prostatic tumorigenesis. Additionally, we show significant upregulation of all three proteins in TRAMP and human invasive prostate tumors. Interestingly, increased MKK4 and MKK7 expression was associated with higher pathologic stage at prostatectomy, although there was no consistent association with time to biochemical recurrence in multivariate analyses. Overall, these findings uncover a fundamental and cell context-specific role for the MAPK signaling cascade during prostatic tumorigenesis.

MATERIALS AND METHODS

Tissue Procurement and Preparation. Formalin-fixed paraffin-embedded human prostatectomy tissue samples were obtained after approval from the University of Chicago Institutional Review

Board. 102 tumor samples were selected by synthetic case control matching from over 500 consecutive radical retropubic prostatectomies performed by two surgeons at our institution between 1995 and 2001. Pertinent patient variables are listed in Table 1. Patient records were evaluated for the presence or absence of biochemical recurrence, defined as the first post-prostatectomy PSA of >0.2 ng/mL. Six tissue microarrays were constructed with triplicate tumor samples from each patient. An additional 20 radical prostatectomy specimens with extensive high grade PIN were selected for separate analysis. Five samples of pathology-verified malignant prostate tissue from the University of Chicago Department of Pathology anonymized tissue bank was flash frozen in OCT for immunoblotting.

Cell Lysis and Immunoblotting. Twenty 20 μm frozen tissue sections were cut from each sample on a cryostat and immediately washed in PBS. The samples were briefly centrifuged at 14,000 rpm (19,000 g), the PBS aspirated and replaced with 250 µL of lysis buffer [10 mM Tris pH 7.5, 1 mM beta-glycerophosphate, 2 mM DTT, 1 mM EDTA, 150 mM NaCl, 0.5 mM NaF, 2 mM $NaVO_4$, 0.1% NP40, 10 µM PMSF, 1% Triton X-100 (w/v), 70 U/ml Aprotinin, and one Complete Protease Inhibitor Cocktail tablet (Roche, Basel, Switzerland)]. Monolayer cell cultures were grown to 80% confluence, washed twice in ice cold PBS, and lysed in above lysis buffer. Cells were collected and passed through a 27 gauge needle. After 30 minutes on ice, the tissue and cell lysate samples were centrifuged at 14,000 rpm (19,000 g) for 10 minutes at 4^{o}C and the supernatant fraction was transferred to a new tube. Protein concentrations were determined using a bicinchoninic (BCA) assay reduction kit (Pierce, Rockford, IL). Sixty µg of total protein from each tissue lysate and 10 µg of total protein from each cell lysate was resolved by SDS-PAGE (12% acrylamide) and transferred to a HyBond ECL nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). Membranes were blocked in TBS-T plus 5% non-fat dry milk (w/v).

Primary antibodies were diluted in blocking solution and incubated with the membrane at 4^{o}C overnight. The primary antibodies and dilutions used are as follows: MKK4 (MEK-4 (K-18), SC-964, Santa Cruz Biotechnology, Santa Cruz, CA, 1:1000 dilution); MKK6 (AF-1604, R&D Systems,

Minneapolis, MN, 1:1000 dilution; MKK7 (BD Transduction Laboratories, 611246, San Jose, CA, 1:1000 dilution), Following a 10 minute wash in blocking solution and 6 washes for 5 minutes each in TBS-T, the membrane was incubated with an HRP-conjugated IgG secondary antibody for one hour, as per the manufacturer's instructions. After an identical series of washes, the HRP-conjugated secondary antibody was detected using the Super Signal West Femto Maximum Sensitivity Chemilluminescence Substrate (Pierce, Rockford, IL). Probed membranes were stripped using Restore Western Blot Stripping Buffer (Pierce, Rockford, IL), washed, and blocked overnight before re-probing. As a loading control, membranes were probed for Actin (Calbiochem, #CP01, 1:10,000) followed by incubation with a goat anti-mouse IgM-peroxidase conjugated secondary antibody (Calbiochem, #JA1200, 1:100,000).

Immunohistochemistry. For immunohistochemical staining, 5-μm tissue sections were washed in xylene and rehydrated in a graded series of ethanol washes. Antigen retrieval for all antibodies was performed in a decloaking chamber (pressure cooker) or a rice steamer chamber in sodium citrate buffer (pH=6). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 15 minutes. The tissue sections were blocked in 5% milk for one hour at room temperature. For MKK4 immunostaining, human tissue sections were incubated at 4 degrees overnight with a mouse monoclonal anti-MKK4 antibody (NCL-MKK4, Novocastra Laboratories, Newcastle upon Tyne, United Kingdom, 1:15 dilution). Mouse tissue sections were incubated at room temperature for 1 hour with a rabbit polyclonal anti-MKK4 antibody (MEK-4 (K-18), SC-964, Santa Cruz Biotechnology, Santa Cruz, CA, 1:150 dilution). Because our previous studies of MKK4 expression in human prostate cancer have exclusively used the Santa Cruz antibody, human tissues were also probed with this antibody and the results of immunostaining with the Novocastra and Santa Cruz antibodies were found to be reasonably correlated (kappa = 0.5030). For MKK6 immunostaining, all tissue sections were incubated at room temperature for 1 hour with a rabbit polyclonal antiserum against MKK6 (AF-1604, R&D Systems, Minneapolis, MN, dilution 1:300). For MKK7 staining, tissues were incubated at room temperature for one hour with a mouse monoclonal anti-MKK7 (BD Transduction

Laboratories, 611246, San Jose, CA, dilution 1:50). Primary antibody incubation was followed by one hour incubation with a biotin-free horseradish peroxidase enzyme–labeled polymer of the EnVision plus detection system (DAKO, Carpinteria, CA). Staining was visualized with diaminobenzidine solution, followed by counterstaining with hematoxylin. For MKK4 staining, the SKOV3ip.1 cell line stably transfected with empty vector or HA-tagged MKK4 was used as a negative and positive control, respectively. For MKK6 and MKK7 staining, human skeletal muscle and renal tissue, respectively, were used as positive controls. Negative controls were performed by using nonimmune rabbit or IgG or antibody diluting solution in place of primary antibody.

Data Analysis. Each tumor or PIN sample was scored for cytoplasmic staining intensity on a 0+ to 3+ scale by a pathologist (TLL). In this system, 0+ staining was defined as a complete lack of visible staining, equal to or less than the negative control tissue. Conversely, 3+ staining was defined as positive cytoplasmic staining, equal to or greater than the appropriate positive control tissue. Surrounding benign glands were also scored when present. Wilcoxon signed-rank test was used to compare immunostaining intensities between neoplastic and normal glands. The immunostaining intensities were correlated with pathologic stage, grade and time to biochemical recurrence data using Spearman correlations and Cox proportional hazard models. Receiver operating characteristic (ROC) analysis was performed to determine the accuracy of the three markers in discriminating between cancer and benign tissues. The global performance of a marker was indicated by the area under the ROC curve. Because of the potential correlation of two tissues from same individual, the 95% confidence interval of area under the ROC curve was calculated using a bootstrapping method. 10,000 replicate samples were drawn with individual Areas under ROC curves were compared using DeLong et al (1988) method.

RESULTS

MKK4, MKK6 and MKK7 are expressed by prostate cancer cell lines and human prostate tumors by immunoblotting. Immunoblots were performed in order to confirm that MKK4, MKK6

and MKK7 proteins are expressed by human prostate carcinoma, as well as to demonstrate the specificity of the antibodies to be used for immunohistochemistry experiments. Immunoblotting of two human prostate cancer cell lines (PC3 and LNCaP) and three flash-frozen, histologically confirmed samples of human prostate carcinoma revealed that MKK4 (MW = 44 kD), MKK6 (MW = 38 kD) and MKK7 (MW = 52 kD) are expressed by prostate cancer cell lines and human prostatic adenocarcinoma, and that the antibodies to be used for the immunohistochemistry experiments were sufficiently specific for these proteins in human tissue samples (Figure 1). Immunoblotting also revealed some variability in the expression levels of these proteins between human tumor samples.

MKK4, MKK6, and MKK7 are weakly expressed in mouse and human benign prostatic epithelial cells by immunohistochemistry. Expression of the MAPK kinases was minimal in the prostates of non-transgenic litter-mate control mice, both in the dorsal-ventral prostates as well as in the coagulating gland/anterior prostate (Figure 2, top panels). Normal human prostatic epithelium adjacent to carcinoma was present for evaluation in 68, 83 and 77 patients for MKK4, MKK6 and MKK7 respectively. All three proteins were only weakly expressed in normal prostatic epithelial cells, with immunostaining intensities less than 1+ on average (Table 3). MKK4 and MKK7 showed predominantly cytoplasmic staining, while MKK6 showed cytoplasmic and nuclear staining in benign prostatic epithelial cells (Figure 2, lower panels). Significantly, the basal cells showed relatively high intensity staining for all three markers, although this was not a consistent finding in every sample. Stromal and smooth muscle staining was minimal for all MAPK kinases, although skeletal muscle bundles showed staining with MKK7, as expected.

MKK4, MKK6, and MKK7 are upregulated in TRAMP and human HGPIN by immunohistochemistry. MAPK kinase immunostaining was also conducted on TRAMP and human high grade PIN (HGPIN) samples. Most TRAMP mice develop PIN lesions by 12 weeks of age, as evidenced by nuclear enlargement, cellular stratification and crowding. MKK4 and MKK6 expression was dramatically increased in the prostatic glands of mice showing the changes of PIN

with nuclear and cytoplasmic localization (Figure 2, top panels). By contrast, in the TRAMP model, MKK7 appeared only modestly upregulated in PIN compared to surrounding benign tissues. In human tissues, all three proteins were significantly upregulated in HGPIN compared to benign glands, with nuclear and cytoplasmic localization (Figure 2, lower panels, Table 2).

MKK4, MKK6 and MKK7 are significantly upregulated in TRAMP and human prostate cancer relative to benign glands. All three MAPK kinases showed upregulation across a spectrum of TRAMP tumors (Figure 3, upper panels) compared to benign prostatic epithelium. In human tumors, MKK4 and MKK6 were generally highly expressed compared to surrounding benign tissues, with highly significant upregulation of both (Figure 3, lower panels, Table 3). MKK7 was also upregulated compared to benign tissues, but the intensity difference was significantly smaller. All three markers showed predominantly cytoplasmic localization with a lesser component of nuclear expression. In order to determine the ability of MKK immunostaining to discriminate between benign and malignant glands, ROC analysis was conducted using the mean staining intensity for each patient. MKK4 and MKK6 immunostaining were highly sensitive and specific markers for human prostatic carcinoma (area under the ROC curve = 0.903 and 0.899 respectively), while MKK7 was a less discriminative marker (area under the ROC curve = 0.694).

Increased expression of MKK4, 6 or 7 is significantly associated with some pathologic and clinical variables, but not correlated with time to biochemical recurrence. Finally, we were interested in whether there was any correlation between clinico-pathologic variables and MAPK kinase staining in the human tumor specimens. Clinical data and follow-up was available on 102 patients. There was no significant correlation with Gleason grade for any of the markers. Higher expression of either MKK4 or MKK7 was significantly correlated with higher pathologic stage at prostatectomy ($p=0.006$ and 0.015 respectively, Table 4). Higher MKK6 expression was moderately correlated with lower preoperative PSA ($p = 0.035$). In multivariate analyses, higher preoperative

PSA ($p = 0.001$), higher Gleason score ($p = 0.02$), and higher pathologic stage ($p<0.001$) all predicted for decreased time to biochemical recurrence, as expected. However, increased expression of MKK4, MKK6 or MKK7 was not independently associated with time to biochemical recurrence ($p=0.46$, $p=0.14$, $p=0.06$ respectively).

DISCUSSION:

The role of SAPK signaling in prostate cancer progression is complex. That MKK4, MKK6 and MKK7 are all *upregulated* in preneoplastic and neoplastic prostate tissues, and that increased expression of MKK4 or MKK7 is associated with *higher* stage tumors is surprising in light of previous work which has identified these proteins as inhibitors of tumor progression in other contexts. One important caveat is that previous studies have shown that the kinase activity of MKK4 is essential for its ability to inhibit metastatic progression [17, 18]. Because we measured *total expression* of MKK4, MKK6 and MKK7, it is not possible to conclude that MKK4, MKK6 and MKK7 *activity* are truly increased in prostatic tumors. However, several recent *in vivo* studies of JNK and p38 activation in both human and TRAMP prostate cancer support the hypothesis that SAPK activity is significantly upregulated in neoplastic prostate tissues. Uzgare et al found that TRAMP PIN and well-differentiated prostate tumors showed elevated p38 activity (by immunoblot and immunohistochemistry) compared to normal prostate tissues [20]. Similarly, in human prostate cancer samples, two recent studies found upregulation of phospho-JNK, as well as p38 activity (as measured by downstream transcription factors Elk-1 and ATF-2) and MKK6 expression [21-23].

Though mounting evidence points to activation of the SAPK signaling cascade during prostate cancer progression, the biological outcome of this activation remains unclear and is likely cell context-specific. Innumerable studies have linked *in vitro* activation of p38 and JNK with apoptosis in prostate cancer cell lines [24-27]. However in TRAMP PIN lesions, *in vivo* p38 activity was associated with cellular proliferation rather than apoptosis[20]. Similarly, though many studies have concluded that activated JNK results in apoptosis in prostate cancer cell lines, others have

suggested that this kinase is a potential target for cell growth[28-30]. While p38 and JNK activity may be associated with local prostate tumor growth, it is quite possible that activation of the same signaling module in a metastatic tumor could be detrimental to tumor cell survival in that context. Overall, it is most likely that the cellular outcome of SAPK activity depends on the integration of numerous extrinsic signals and intracellular signaling cascades. Recent in vivo studies have suggested that the *balance* of p38 and ERK activity is critical in determining the proliferative fate of tumor cells[31, 32]. The fact that increased levels MKK4, MKK6 and MKK7 expression were not independently associated with prognosis in prostate cancer in our study may reflect the significance of such a balance.

While SAPK signaling may play a key role in prostate cancer progression, the upregulated expression of MKK4, MKK6 and MKK7 in prostate lesions may also represent a cellular response to stress during the transformation process. We found that MAPK kinase expression is both increased in intraepithelial prostatic neoplasia and later associated with local prostate tumor progression. In other contexts, expression of SAPK signaling components is also temporally dynamic during tumor progression, perhaps reflecting changing levels of cellular stress. In the stomach, MKK4 expression is significantly increased in gastric intestinal metaplasia (generally a response to inflammation) as well as in gastric dysplasia (a pre-neoplastic lesion) compared to surrounding normal glands[33]. Yet in gastric cancers, increasing MKK4 expression was associated with better patient survival, suggesting that MKK4 was progressively downregulated in more advanced tumors[15, 34]. This pattern of expression suggests that even before neoplastic transformation takes place, MAPK kinase levels may be increased as a response to surrounding inflammation or other cellular stressors. However, with tumor progression, the MAPK kinase levels may continue to rise (as in prostate cancer) or fall, perhaps reflecting a defective response to external stress (as in gastric tumors).

Ultimately, our finding that multiple components of the SAPK signaling cascade are dramatically upregulated during prostatic tumorigenesis, both in a mouse model as well as in human tissues, has

significant diagnostic and therapeutic ramifications. At a minimum, proteins in the SAPK signaling cascade provide an attractive target for future biomarkers and immunohistochemical tests that may play an important role in diagnosis of prostate tumors. Given the conflicting results *in vitro*, future studies should be aimed at pinpointing the biological effects of JNK and p38 activation *in vivo*, both in localized as well as metastatic prostate tumors. The availability of small molecule inhibitors of p38 and JNK activity will be particularly helpful in these studies and may be of future therapeutic benefit. In the end, identification of the signaling cascades that are differentially activated during prostatic tumorigenesis is a crucial step in the search for future molecular targets in this disease.

Tables:

Table 1: Patient characteristics

Number of patients	102
Median age in years (range)	62 (42-74)
Pre-operative PSA, n (%)	
< 4	8 (7.6%)
4-9.9	69 (65.7%)
10-19.9	21 (20.0%)
≥ 20	7 (6.7%)
Gleason score, n (%)	
≤ 6	45 (42.5%)
7	53 (50.0%)
≥ 8	8 (7.5%)
Pathologic stage, n (%)	
T2	60 (56.6%)
T3	39 (36.8%)
T4	7 (6.6%)
Surgical margin status	
Negative	89 (84.0%)
Positive	17 (16.0%)
Median follow up in months (range)	36 (3-119)
Recurrence	
Yes	42 (40.4%)
No	62 (59.6%)
2-year recurrent rate	21.30%
5-year recurrent rate	49.20%

Table 2. IHC staining scores in normal human prostate and HGPIN tissues

Protein	# of patients	Mean staining intensity (SD)		p-value
		normal tissue	PIN	
MKK4	20	0.3 (0.4)	2.4 (1.0)	0.0001
MKK6	21	1.0 (0.2)	2.6 (0.5)	<0.0001
MKK7	20	1.0 (0.5)	2.6 (0.5)	0.0001

Table 3: IHC staining scores in normal human prostate and cancer tissues

Protein	# of patients	Mean staining intensity (SD)		p-value
		normal tissue	cancer	
MKK4	68	0.7 (0.5)	1.9 (0.7)	<0.0001
MKK6	83	0.9 (0.5)	2.0 (0.7)	<0.0001
MKK7	77	0.4 (0.5)	0.8 (0.7)	<0.0001

Table 4: p-values and correlation coefficients for MKK staining and pathologic/clinical variables

	p-value (correlation coefficient)		
Protein	**PSA**	**Gleason score**	**Pathologic stage**
MKK4	0.61	0.15	**0.006** (0.28)
MKK6	**0.035** (-0.22)	0.39	0.44
MKK7	0.61	0.82	**0.015** (0.25)

Figures:

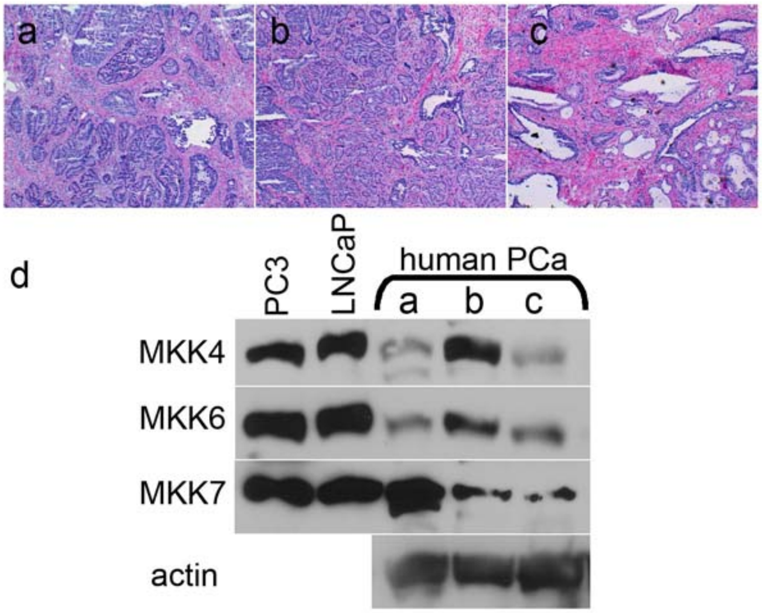

Figure 1: *MKK4, MKK6 and MKK7 proteins are expressed in human prostate cancer cell lines and frozen tumor tissue.* Frozen sections were prepared from human prostate cancer tissues, and these tissues were subsequently lysed to obtain protein for immunoblotting (all at 40x magnification). **(a)** Gleason grade 7(4+3) **(b)** Gleason grade 7(3+4) and **(c)** Gleason grade 6(3+3) tumors. **(d)** Immunoblotting of 60 µg of protein from each of the above tissues reveals that MKK4 (44 kD), MKK6 (38 kD) and MKK7 (52 kD) are variably expressed in human prostate tumors as well as in 10 µg of lysate from human prostate cancer cell lines (PC3 and LNCaP) and that the antibodies to be used for immunohistochemistry experiments are reasonably specific for these proteins.

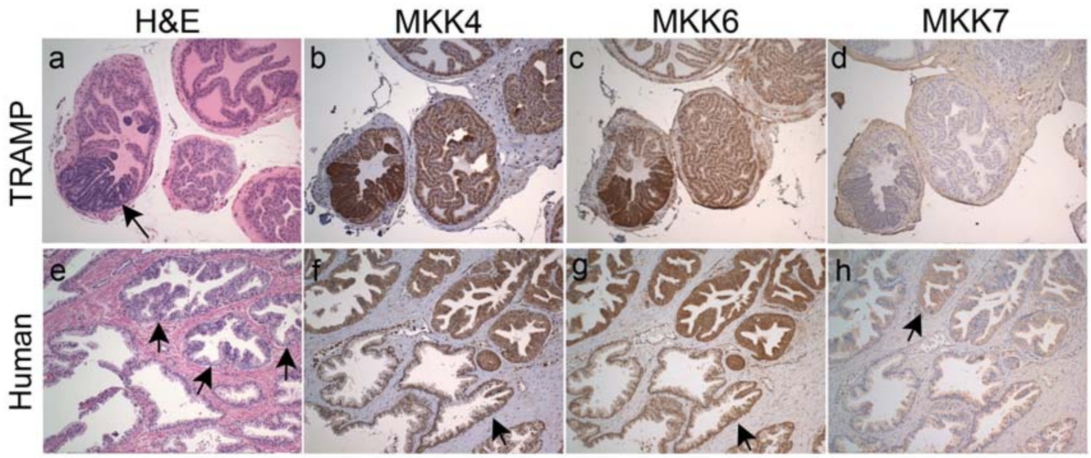

Figure 2: *Increased MKK4, MKK6 and MKK7 expression in TRAMP and human prostatatic intraepithelial neoplasia (PIN).* Representative PIN lesions in both TRAMP and human tissues are characterized by a hyperplastic epithelium, with enlarged nuclei and prominent nucleoli (**a**, **e**, 100x magnification, arrows indicate PIN and high grade PIN in TRAMP and human tissues respectively). MKK4 and MKK6 are overexpressed in TRAMP and human HGPIN relative to surrounding normal prostate epithelial cells (**b**, **c**, **f**, **g** 100x, arrows indicate high MKK4 and MKK6 expression in basal cells of benign human glands). MKK7 is weakly expressed in TRAMP PIN and focally overexpressed in human PIN (arrow) (**d**, **h**, 100x).

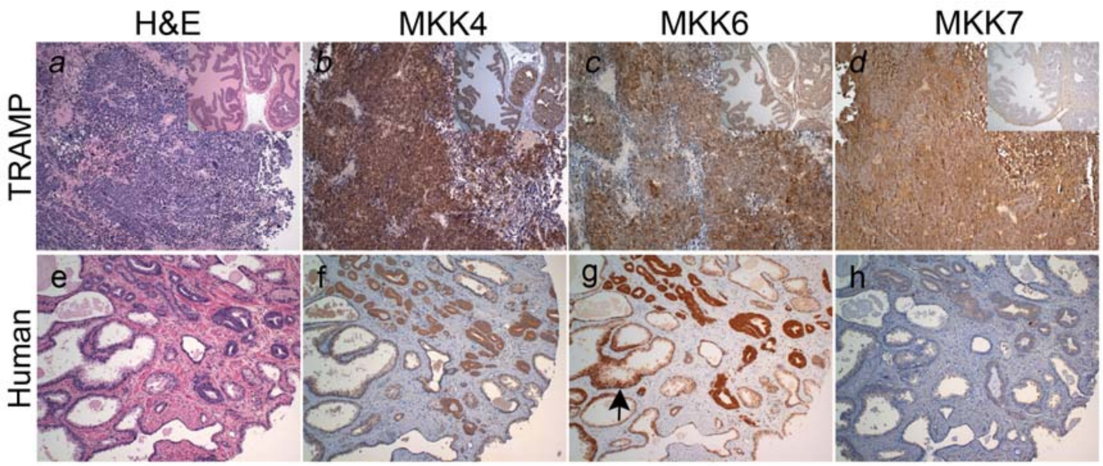

Figure 3: *Increased MKK4, MKK6 and MKK7 expression in TRAMP and human prostate tumors.* A representative poorly differentiated TRAMP prostate tumor shows increased MKK4, MKK6 and MKK7 expression relative to nearby benign glands from the same animal (inset) (**a**, **b**, **c**, **d** 100x). A representative human prostate tumor (Gleason 6) from the tissue microarray also shows dramatic upregulation of MKK4 and MKK6 with moderate expression of MKK7 in infiltrating malignant glands relative to surrounding, larger benign glands (**e**, **f**, **g**, **h** 100x). A focus of PIN with high expression of MKK6 (**g**, arrow) is coincidentally present.

References:

1. Kennedy, N. J., Davis, R. J.: Role of JNK in tumor development. Cell Cycle, **2:** 199, 2003

2. Schreiber, M., Kolbus, A., Piu, F. et al.: Control of cell cycle progression by c-Jun is p53 dependent. Genes Dev, **13:** 607, 1999

3. Sun, P., Yoshizuka, N., New, L. et al.: PRAK Is Essential for ras-Induced Senescence and Tumor Suppression. Cell, **128:** 295, 2007

4. Tront, J. S., Hoffman, B., Liebermann, D. A.: Gadd45a Suppresses Ras-Driven Mammary Tumorigenesis by Activation of c-Jun NH2-Terminal Kinase and p38 Stress Signaling Resulting in Apoptosis and Senescence. Cancer Res, **66:** 8448, 2006

5. Kennedy, N. J., Sluss, H. K., Jones, S. N. et al.: Suppression of Ras-stimulated transformation by the JNK signal transduction pathway. Genes Dev, **17:** 629, 2003

6. Cazillis, M., Bringuier, A. F., Delautier, D. et al.: Disruption of MKK4 signaling reveals its tumor-suppressor role in embryonic stem cells. Oncogene, **23:** 4735, 2004

7. Su, G. H., Hilgers, W., Shekher, M. C. et al.: Alterations in pancreatic, biliary, and breast carcinomas support MKK4 as a genetically targeted tumor suppressor gene. Cancer Res, **58:** 2339, 1998

8. Su, G. H., Song, J. J., Repasky, E. A. et al.: Mutation rate of MAP2K4/MKK4 in breast carcinoma. Hum Mutat, **19:** 81, 2002

9. Teng, D. H., Perry, W. L., 3rd, Hogan, J. K. et al.: Human mitogen-activated protein kinase kinase 4 as a candidate tumor suppressor. Cancer Res, **57:** 4177, 1997

10. Nakayama, K., Nakayama, N., Davidson, B. et al.: Homozygous deletion of MKK4 in ovarian serous carcinoma. Cancer Biol Ther, **5:** 630, 2006

11. Chekmareva, M. A., Kadkhodaian, M. M., Hollowell, C. M. et al.: Chromosome 17-mediated dormancy of AT6.1 prostate cancer micrometastases. Cancer Res, **58:** 4963, 1998

12. Yamada, S. D., Hickson, J. A., Hrobowski, Y. et al.: Mitogen-activated protein kinase kinase 4 (MKK4) acts as a metastasis suppressor gene in human ovarian carcinoma. Cancer Res, **62:** 6717, 2002

13. Yoshida, B. A., Dubauskas, Z., Chekmareva, M. A. et al.: Mitogen-activated protein kinase kinase 4/stress-activated protein/Erk kinase 1 (MKK4/SEK1), a prostate cancer metastasis suppressor gene encoded by human chromosome 17. Cancer Res, **59:** 5483, 1999

14. Xin, W., Yun, K. J., Ricci, F. et al.: MAP2K4/MKK4 expression in pancreatic cancer: genetic validation of immunohistochemistry and relationship to disease course. Clin Cancer Res, **10:** 8516, 2004

15. Cunningham, S. C., Kamangar, F., Kim, M. P. et al.: MKK4 status predicts survival after resection of gastric adenocarcinoma. Arch Surg, **141:** 1095, 2006

16. Stark, A. M., Tongers, K., Maass, N. et al.: Reduced metastasis-suppressor gene

mRNA-expression in breast cancer brain metastases. J Cancer Res Clin Oncol, **131:** 191, 2005

17. Hickson, J. A., Huo, D., Vander Griend, D. J. et al.: The p38 kinases MKK4 and MKK6 suppress metastatic colonization in human ovarian carcinoma. Cancer Res, **66:** 2264, 2006

18. Vander Griend, D. J., Kocherginsky, M., Hickson, J. A. et al.: Suppression of metastatic colonization by the context-dependent activation of the c-Jun NH2-terminal kinase kinases JNKK1/MKK4 and MKK7. Cancer Res, **65:** 10984, 2005

19. Kim, H. L., Vander Griend, D. J., Yang, X. et al.: Mitogen-activated protein kinase kinase 4 metastasis suppressor gene expression is inversely related to histological pattern in advancing human prostatic cancers. Cancer Res, **61:** 2833, 2001

20. Uzgare, A. R., Kaplan, P. J., Greenberg, N. M.: Differential expression and/or activation of P38MAPK, erk1/2, and jnk during the initiation and progression of prostate cancer. Prostate, **55:** 128, 2003

21. Ricote, M., Garcia-Tunon, I., Bethencourt, F. et al.: The p38 transduction pathway in prostatic neoplasia. J Pathol, **208:** 401, 2006

22. Royuela, M., Arenas, M. I., Bethencourt, F. R. et al.: Regulation of proliferation/apoptosis equilibrium by mitogen-activated protein kinases in normal, hyperplastic, and carcinomatous human prostate. Hum Pathol, **33:** 299, 2002

23. Magi-Galluzzi, C., Montironi, R., Cangi, M. G. et al.: Mitogen-activated protein kinases and apoptosis in PIN. Virchows Arch, **432:** 407, 1998

24. Cho, S. D., Ahn, N. S., Jung, J. W. et al.: Critical role of the c-JunNH2-terminal kinase and p38 mitogen-activated protein kinase pathways on sodium butyrate-induced apoptosis in DU145 human prostate cancer cells. Eur J Cancer Prev, **15:** 57, 2006

25. Curtin, J. F., Cotter, T. G.: Anisomycin activates JNK and sensitises DU 145 prostate carcinoma cells to Fas mediated apoptosis. Br J Cancer, **87:** 1188, 2002

26. Tanaka, Y., Gavrielides, M. V., Mitsuuchi, Y. et al.: Protein kinase C promotes apoptosis in LNCaP prostate cancer cells through activation of p38 MAPK and inhibition of the Akt survival pathway. J Biol Chem, **278:** 33753, 2003

27. Wu, J., Haugk, K., Plymate, S. R.: Activation of pro-apoptotic p38-MAPK pathway in the prostate cancer cell line M12 expressing a truncated IGF-IR. Horm Metab Res, **35:** 751, 2003

28. Godoy-Tundidor, S., Cavarretta, I. T., Fuchs, D. et al.: Interleukin-6 and oncostatin M stimulation of proliferation of prostate cancer 22Rv1 cells through the signaling pathways of p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase. Prostate, **64:** 209, 2005

29. Koochekpour, S., Sartor, O., Hiraiwa, M. et al.: Saposin C stimulates growth and invasion, activates p42/44 and SAPK/JNK signaling pathways of MAPK and upregulates uPA/uPAR expression in prostate cancer and stromal cells. Asian J Androl, **7:** 147, 2005

30. Uzgare, A. R., Isaacs, J. T.: Enhanced redundancy in Akt and mitogen-activated protein kinase-induced survival of malignant versus normal prostate epithelial cells. Cancer Res, **64:** 6190, 2004

31. Aguirre-Ghiso, J. A., Ossowski, L., Rosenbaum, S. K.: Green fluorescent protein tagging of extracellular signal-regulated kinase and p38 pathways reveals novel dynamics of pathway activation during primary and metastatic growth. Cancer Res, **64:** 7336, 2004

32. Ranganathan, A. C., Adam, A. P., Aguirre-Ghiso, J. A.: Opposing roles of mitogenic and stress signaling pathways in the induction of cancer dormancy. Cell Cycle, **5:** 1799, 2006

33. Cunningham, S. C., Kamangar, F., Kim, M. P. et al.: Claudin-4, mitogen-activated protein kinase kinase 4, and stratifin are markers of gastric adenocarcinoma precursor lesions. Cancer Epidemiol Biomarkers Prev, **15:** 281, 2006

34. Wu, C. W., Li, A. F., Chi, C. W. et al.: Human gastric cancer kinase profile and prognostic significance of MKK4 kinase. Am J Pathol, **156:** 2007, 2000